DOI 10.1007/s10517-020-04887-6
Bulletin of Experimental Biology and Medicine, Vol. 169, No. 3, July, 2020 IMMUNOLOGY AND MICROBIOLOGY

Bactericidal Activity of Liposomal Form of Lytic Mycobacteriophage D29 in Cell Models of Tuberculosis Infection *In Vitro*

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 169, No. 3, pp. 334-337, March, 2020
Original article submitted September 12, 2019

The use of lytic mycobacteriophages to treat tuberculosis under conditions of acquired resistance to anti-tuberculosis drugs is one of the most practical ways to improve the effectiveness of therapy and reduce the spread of this disease. We studied the efficacy of antimycobacterial action of mycobacteriophage D29 encapsulated into 400-nm liposomes in cell models of tuberculosis infection *in vitro*. The antimycobacterial action of lytic mycobacteriophage D29 used in free or liposome-encapsulated forms was demonstrated on cell models of intracellularly infected RAW264.7 macrophages and tuberculous granuloma formed by human blood mononuclear cells. The experiments demonstrated pronounced advantage of liposomal form of mycobacteriophage according to the criteria of their penetration into macrophages and lysis of *Mycobacterium tuberculosis* in culture.

Key Words: *mycobacteria tuberculosis; mycobacteriophage; macrophages; mononuclears; liposomes*

The global plan of radical (by 90%) reduction of the incidence of tuberculosis by 2035 announced by WHO greatly depends on the development of innovative treatment methods that can essentially shorten the duration of treatment of this disease and reduce the spread of drug resistance to the basic anti-tuberculosis drugs.

The biological nature of *Mycobacterium tuberculosis* (MBT) with 24-h DNA doubling time determines the duration of antibiotic therapy and appearance of drug-resistant MBT strains. The duration of chemotherapeutic courses in case of multiple drug resistance (resistance to isoniazid and rifampicin) attains 1.5-2.0 years, which reduces patient's compliance to anti-tuberculosis therapy.

A fundamentally new approach to the problem of tuberculosis is inclusion of lytic mycobacteriophages into treatment protocols because they are highly specific and can overcome drug resistance of MBT [3,4,6,8]. However, the use of mycobacteriophages in the therapy of tuberculosis is impeded by some hurdles, the major challenges being mycobacteriophage delivery into the areas of infiltrative tuberculosis inflammation and the risk of formation of specific antibodies that could neutralize the phage particles [10]. In light of this, the use of mycobacteriophages encapsulated into liposomes seems promising [7,9].

Bactericidal effect of lytic mycobacteriophage D29 against MBT was previously demonstrated by us on the model of transplantable line of macrophages infected with MBT *in vitro* [1].

Here we studied the bactericidal action of mycobacteriophages D29 encapsulated into 400-nm liposomes on cell models of tuberculosis infection *in vitro*.

MATERIALS AND METHODS

Preparation of lytic mycobacteriophages. We used lytic mycobacteriophage D29 kindly provided by Prof.

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Gr. Hatful (Pittsburgh Bacteriophage Institute, USA). Mycobacteriophages were expanded during culturing of *M. smegmatis* (MC^2 155) in a liquid Middlebrook 7H9 culture medium with 10% OADC and 1 mM $CaCl_2$ [1], which allowed obtaining phage preparations with a concentration of 10^{10}-10^{11} plaque-forming units (PFU, *i.e*. lysis zones on the lawn of agar culture of *M. smegmatis*). Isolation and purification of phage particles of *M. smegmatis* bacterial lysates were carried out by ion exchange chromatography on a Q Sepharose column [2].

Preparation of liposomal lytic mycobacteriophages. Purified mycobacteriophage preparation with a concentration of $\geq 10^9$ PFU/ml in phage buffer (3 ml) was shaken in a flask for 5-10 min after evaporation in a rotary evaporator of the phospholipid film consisting of 40 mg egg lecithin (Lipoid), 8 mg cholesterol, and 8.2 mg Tween-80. The obtained suspension was extruded through a 5-μ filter followed by 20-fold extrusion through 0.4-μ filters. Finally, mycobacteriophage fraction included into liposomes was separated by 30-min centrifugation at 20,000 rpm.

The fraction of liposome-encapsulated D29 was evaluated by quantitative real-time PCR of mycobacteriophage DNA using D29 primers AGCCGATCAGAAGCACGGGC (F) and AGCGGCTCTTAGGAGGGGCC (R) and FAM-labeled probe AGCCACGAACTCGCGACCCACGG. The fraction of mycobacteriophages included into liposomes was ≥15%

Cell models of tuberculosis infection. The lytic (antibacterial) effect of liposome-encapsulated D29 was examined on two cell models: intracellularly infected RAW264.7 (ATCC) macrophage culture and *in vitro* tuberculous granuloma [5] formed by human peripheral blood mononuclears in the presence of MBT.

In the first model, RAW 264.7 cells were cultured in a CO_2 incubator at 37°C in 6-well plates with RPMI-1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, penicillin-streptomycin, Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA), MEM Vitamins, and 1 mM sodium pyruvate (Gibco). The cells were infected over 1 day with growing MBT of the virulent H37Rv strain at a ratio of 15 MBT per macrophage (calculated by quantitative analysis of MTB DNA using the single-copy RegX3 gene). To separate macrophages from non-phagocytized MBT, the macrophages were washed with culture medium and centrifuged in a Ficoll-Paque density gradient; the interface layer was collected and the cells were washed with physiological saline and culture medium. Then, macrophages infected with MTB were cultured for 24 h in the same medium with addition of 100 μl free D29 (10^8 PFU/ml), 100 μl liposome-encapsulated D29 (10^8 PFU/ml), or 100 μl culture medium (control wells). After that, macrophages were scrapped from the wells in a volume of 200 μl per well, twice rapidly frozen and thawed, and transferred to the dishes (100 μl per dish) with Middlebrook 7H10 culture medium added with OADC Growth Supplement. The cultures were analyzed in 3 weeks. Penetration of liposomal and extraliposomal mycobacteriophages into macrophages was assessed with quantitative PCR.

Peripheral blood mononuclears were isolated by centrifugation in a Ficoll-Paque density gradient and washed in physiological saline and RPMI-1640 medium, and then cultured in the same medium with growth supplements in a CO_2 incubator at 37°C and 5% CO_2 in 24-well plates (500 μl/well, 1.0-1.2×10^6 cell/ml).

The model of tuberculous granuloma [5] was developed by culturing peripheral blood mononuclears from patients with latent or active tuberculosis at 6:1 eukaryotes-MBT ratio.

RPMI-1640 medium with the above supplements was refreshed every 2 days. Cultivation was monitored under a Leica inverted microscope with a Leica DFC 420 digital camera at 200×. Granulomas were formed on days 13-15 (Fig. 1).

After formation of granulomas, the medium was refreshed, and 50 μl free or liposomal mycobacteriophages (10^9 PFU/ml) were added into every 3 wells; nutrient medium was added to control wells.

In 24 h, the granuloma cells were washed once with the medium, scraped in a volume of 200 μl, and subjected to two freezing—thawing cycles. Then the specimens (100 μl) were plated onto the dishes with Middlebrook 7H10 culture medium added with OADC Growth Supplement. Two experiments were conducted by this protocol.

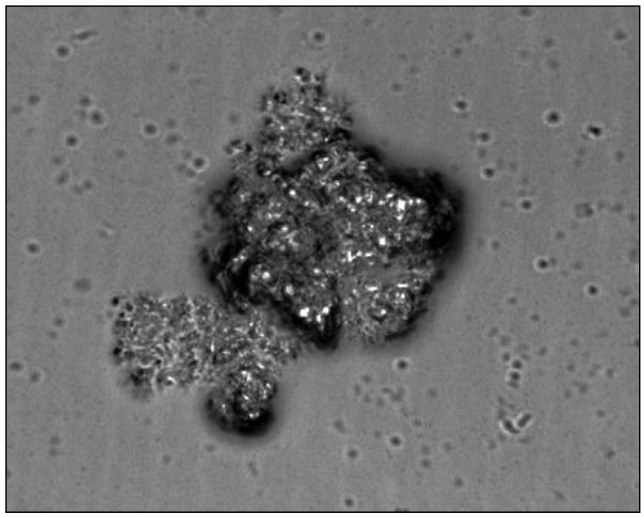

Fig. 1. Formation of granuloma with destructive focus in the center. Light microscopy, ×200.

RESULTS

The results of assessment of the antimycobacterial action of free or liposomal mycobacteriophages on the model of intracellularly infected RAW 264.7 macrophages are presented in Table 1. The antibacterial effect of liposome-encapsulated D29 was significantly higher than that of free mycobacteriophage.

Quantitative real-time PCR showed that the gene copy number assessing amount of DNA in the phages encapsulated into liposomes and detected in the infected macrophages was 1.1×10^6 when the free mycobacteriophages were employed, whereas the similar values were 8.4×10^6 and 6.4×10^6 in two specimens prepared with liposomal form of mycobacteriophages. Thus, effectiveness of penetration of liposome-encapsulated D29 into macrophages was 6.7-fold higher. The results of evaluation of the efficiency of mycobacterium inhibition by free or liposome-encapsulated lytic mycobacteriophage D29 on the model of tuberculous granuloma in vitro are shown in Table 2.

During overt destruction of the cells in formed tuberculous granuloma resulting from the development of infection, the number of MBT in the control and in experiments with free mycobacteriophages did not significantly differ (Fig. 2). In contrast, effectiveness of liposome-encapsulated D29 was significantly higher in both experiments ($p<0.001$). The results of experiment No. 2 are presented in Figure 3.

Thus, we showed that the use of liposomal form of lytic mycobacteriophage D29 increases penetration of its particles into infected macrophages by 6-8 times. Moreover, this liposomal form produces significantly greater antimycobacterial effect in comparison with free mycobacteriophages. These findings open new vista to the development and clinical use of liposomal forms of lytic mycobacteriophages in tuberculosis treatment.

TABLE 1. Antimycobacterial Action of Free and Liposomal Mycobacteriophage D29 on the Model of Intracellularly Infected Macrophages (m±SE)

Group	Mean number of MBT colonies
Control MBT	62.0±2.6
Free mycobacteriophage D29	17±1**
Liposomal mycobacteriophage D29	7.0±0.3*

Note. *$p<0.05$, **$p<0.01$ in comparison with the control (Student's *t* test).

TABLE 2. Antimycobacterial Action of Free and Liposomal Mycobacteriophage D29 on the Model of Tuberculosis Granuloma *In Vitro* (m±SE)

Group	Mean number of MBT colonies
Control	>1000
Free mycobacteriophage D29	583±60
Liposomal mycobacteriophage D29	64±7+

Note. +$p<0.001$ in comparison with free mycobacteriophage D29.

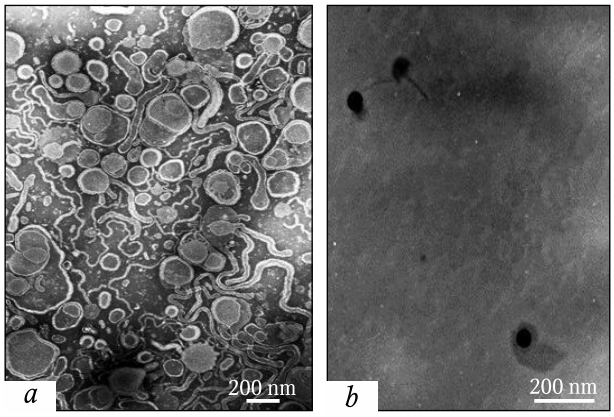

Fig. 2. Electron microscopy of liposomes and liposomal mycobacteriophage, ×50,000. *a*) Phage-free bilayer liposomes. Negative contrast with 1% uranyl-acetate; *b*) phage head encapsulated in a liposome. Positive contrast.

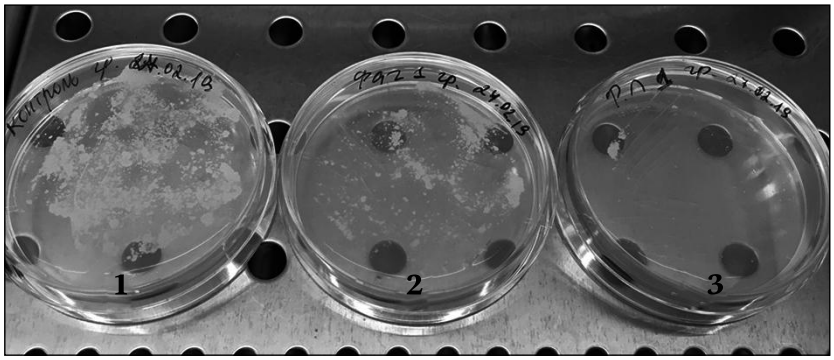

Fig. 3. Results of inoculation of granuloma samples after treatment with mycobacteriophages. *1*) Control; *2*) free D29; *3*) liposome-encapsulated mycobacteriophages.

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